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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Lo *et al* Attorney Docket No: JAK-PT001.1

Application No.: 09/872,063 Group: 1655

Filed: June 1, 2001 Examiner: Jeanine E. Goldberg

For: NON-INVASIVE PRENATAL DIAGNOSIS

DECLARATION UNDER RULE 132

Identification of Declarant

1. I, Y-M. DENNIS LO, declare that I am one of the inventors of the above-identified application and am a professor of the Chinese University of Hong Kong, Department of Chemical Pathology, Prince of Wales Hospital, Shatin, New Territories, Hong Kong.

Purpose of this Declaration

2. I have been informed that an official letter has been issued on this application, in which the Patent Office examiner has rejected the claims on the grounds (among others) that the specification is not enabling in relation to the detection of fetal DNA during early pregnancy and to the detection of single gene disorders. The purpose of this Declaration is to address these concerns.

References to the Specification of the above Patent Application

3. In the declaration, I refer to the paragraph numbers of the printed application US 2001/0051341 A1.

Detection of fetal DNA in the First Trimester of Pregnancy

4. The examiner has referred to statements made in my paper Lo *et al.*, New England Journal of Medicine **339** (24) 1734-1738 (December 10, 1998), at page 1736 r.h column and Lo, Annals of Medicine **31**, 308-312 (1999), at page 310 r.h. column. These statements can be read as implying that prenatal diagnosis in the first trimester of pregnancy (7 to 14 weeks) by the

method of the invention may be unreliable. I now believe that these statements, made in refereed journals, are over-cautious and that it was fully justified to take a wider view of the applicability of my work when the patent application was first prepared. Thus, I believe that those familiar with DNA amplification techniques would be able to achieve useful results from samples taken during the first trimester of pregnancy. My reasons are as follows:

(a) Referring to the patent application itself, Figures 4A to 4L and the associated description at paragraph [0152], it will be seen that the SRY gene (from male fetuses) was detected within the first trimester in all 12 samples:

11 weeks	-	4A	12 weeks	-	4G
7 & 11 weeks	-	4B	8 & 14 weeks	-	4H
7 & 12 weeks	-	4C	8 & 12 weeks	-	4I
8 & 12 weeks	-	4D	8 & 12 weeks	-	4J
7 & 12 weeks	-	4E	8 & 14 weeks	-	4K
7 & 12 weeks	-	4F	8 weeks	-	4L

(b) Figures 4A-4L are reproduced on page 773 of the later-published paper Lo *et al.*, American Journal of Human Genetics **62**, 768-775 (April 1, 1998). In this paper it is stated on page 773 l.h. column:

> "Second, we were able to detect fetal SRY sequences as early as the 7th week of gestation, thus indicating that fetal genetic analysis in maternal plasma/serum could be used in the first trimester."

This statement could have been made in the patent application, since the data on which it is based are the same. Other competent workers in the field must have recognised this: Faas *et al.*, The Lancet 352, 1196 (October 10, 1998), refer to this American Journal of Human Genetics paper as reference (5) and say (last sentence of their paper):

> "Lo and colleagues showed that fetal DNA can be detected in the maternal plasma in the first trimester, which makes the plasma PCR promising in prenatal tests early in pregnancy".

(c) Sekizawa *et al.*, Clinical Chemistry **47**, 1856-1858 (2001) describe a large trial, on 302 pregnant women between 7 and 16 weeks of gestation, of whom 275 were in the range 7 to 14 weeks. Male fetuses were detected by extracting DNA from maternal plasma (by the same method as in the patent application) and carrying out real time PCR (using a similar method to that described in the present patent application. An accurate diagnosis was made in all except 4 of the 133 women carrying a male fetus and in all 142 women carrying a female fetus (see table on page 1857).

The authors conclude (last paragraph):

> " In conclusion, this is the first description of the diagnostic accuracy of fetal gender determination from a large number of maternal plasma samples obtained at early gestation. Because this test is non-invasive and highly accurate, it can be used as a valuable first step in various clinical settings."

Since the techniques used were of an ordinary kind, closely resembling those described in the patent application, this paper proves to me that the present invention could have been carried out at the date of the present invention in the first trimester of pregnancy.

(d) Similarly, Honda *et al.*, Human Genetics **110**, 75-79 (2002), carried out gender determination on the fetuses of 81 pregnant women by extracting DNA from maternal serum (by the same method as in the patent application) and carrying out real time PCR (using a similar method to that described in the present patent application.

Referring to Fig 1 and Table 1 on page 77, but considering only the first trimester, namely weeks 7-14, an accurate diagnosis was made in all 35 women carrying a male fetus (there were 5 women sampled in weeks 5 and 6 under the diagnosis was not wholly successful). For the 40 women carrying a female fetus, the diagnosis was, again, 100% accurate. Honda *et al.* discuss at length the sensitivity of the detection, saying (page 77, l.h. column, bottom)

> "Indeed, all the fetal DNA concentrations in maternal serum obtained from pregnant women after the 7^{th} week were over

the detection limit of fetal DNA concentration for detection by our real-time quantitative PCR of the SRY gene"

The statement confirms that there is sufficient fetal DNA present in the maternal serum for detection by conventional PCR. These detectable quantities of DNA could be utilised by those skilled in the art to detect any target sequence within the DNA, just by the use of the appropriate primer sequence in relation to that target. Therefore, it appears to me that the invention has been fully "enabled", as of the invention date, without limitation as to the chromosomal locus of the DNA detected.

(e) Amicucci *et al.,* Clinical Chemistry **46**, 301-302 (2000), carried out the prenatal diagnosis of fetal myotonic dystrophy by using maternal plasma. Myotonic dystrophy is autosomal, i.e. the relevant genes are not carried on the sex-determining chromosomes (X,Y). The genetic mutation responsible involves extensive CTG repeats. The mother was not suffering from the disease. Conventional PCR was carried out using primers for amplifying the portion of the gene carrying the CTG repeats and, in a second round of PCR, Y chromosome-specific primers gave a clear result. The paternal DNA showed 70 CTG repeats, the maternal peripheral blood lymphocyte DNA only 5 CTG repeats and the maternal plasma 150 CTG repeats. Under the conditions used, the maternal peripheral blood does not contain sufficient fetal cells to give any signal and therefore gives a measure of the maternal CTG repeats only. These results demonstrate that PCR carried out on another genetic locus than the Y chromosome can lead to successful diagnosis of an inherited disease, from samples taken during the first trimester of pregnancy.

Detection of Single Gene Disorders

5. The present patent application is based on the surprising finding that the fractional concentration of fetal DNA in maternal serum or plasma is remarkably high. Experimentally, I and my colleagues found that the fractional concentration in early pregnancy (11 to 17 weeks) ranged from 0.39% to 11.9% (mean 3.4%) see paragraph [0150] of the patent application. This is a much higher concentration than the corresponding fractional concentration of fetal cells in maternal blood. For the latter, Nakagome *et al.*, American Journal of Medical Genetics **40**, 506-508 (1991), have shown that fetal cells are present in the blood leukocyte fraction of maternal blood at a fractional concentration of less than 1 in 25,000 (i.e. 0.004%). Similarly, Gänshirt-

Ahlert *et al.*, Clinical Genetics **38**, 38-43 (1990), have shown that the ratio of fetal to maternal DNA in the cellular fraction of blood is less than 1 in 5,000 (i.e. 0.02%). The patent application paragraph [0155] shows that the mean concentration of fetal DNA in maternal plasma during the first trimester is 24.5 cell equivalents/ml. This is more than 20 times the level (1.2 cell equivalents/ml) reported by Bianchi *et al*, American Journal of Human Genetics **61**, 822-829 (1997), for second trimester whole maternal blood samples. The data by Bianchi et al can also be expressed in terms of fractional concentrations. It is well established that the normal range of white cell count in peripheral blood is between 4,400 to 11,000/μl (for example, see http://www.bioscience.org/atlases/clinical/hematol/hematol.htm), i.e., corresponding to $4.4x10^6$ to $11x10^6$/ml. Thus, the mean fractional concentration of fetal cells in maternal blood, as deduced by the data from Bianchi *et al.* would be from $1.2/4.4x10^6$ to $1.2/11x10^6$, corresponding to $2.73x10^{-5}$% to $1.09x10^{-5}$%. When these values are compared with the above-mentioned mean fractional concentration of fetal DNA in maternal plasma in early pregnancy of 3.4%, one can see that plasma offers a fractional concentration of fetal DNA that is 124,000 to 311,000 times higher than the corresponding concentration in maternal whole blood samples. In summary, the fundamental teachings of the present patent application are (1) that the detection of fetal DNA in maternal plasma or serum is easier and more reliable than in whole peripheral blood (or the cellular fraction thereof), and (2) that sufficient DNA is present in the maternal serum and plasma, even during the first trimester of pregnancy, to enable the detection of fetal DNA from any chromosomal locus.

6. Despite the rarity of fetal cells in the cellular fraction of maternal whole blood, numerous non-Y chromosome fetal genes or DNA sequences have been detected. Examples include:

(a) DNA polymorphisms linked to the cystic fibrosis transmembrane conductance regulator (CFTR) gene (an autosomal gene responsible for cystic fibrosis) see Lo *et al.*, Annals of the New York Academy of Science **731**, 204-213 (1994). The CFTR gene is on chromosome 7. The most frequent type of defect is a deletion of the nucleotides CTT between two codons, causing deletion of one amino acid. The defect is recessive. Those who have it on only one of their two chromosomes are carriers, while those who have it on both chromosomes will show the disease

phenotype. The defect is linked to a restriction fragment length polymorphism known as KM19/*Pst*I.

This paper shows that this polymorphism can be detected by a method of PCR depicted in Figure 3 on page 208. Its basis is that within a previously characterised family a paternal allele associated with the defective CFTR remains uncleaved after a restriction enzyme digest, whereas the normal allele is cleaved. When the digest products are amplified by "ARMS" PCR, the uncleaved, paternally inherited allele is detected. "ARMS is a technique explained on page 205. It involves ensuring that the primer matches the paternal allele, but has a 3'-terminal mismatch with the maternal alleles. This results in discriminatory favourable amplification of the paternal allele relative to the maternal.

As may be seen, the experimental results were successful, the combination of the restriction enzyme digestion and a round of "ARMS" PCR being effective to avoid false positives (page 209, middle).

(b) The same paper further describes detection of alleles 5' (upstream) of the delta-globin gene. This is a highly polymorphic region, which enables two pairs of PCR primers to be constructed. Double "ARMS" PCR is then carried out, each pair of primers having mismatches at their 3'-ends corresponding to a DNA polymorphism. Again, the diagnosis was reasonably successful. See Table 2 on page 211.

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The test (a) and diagnosis (b) above were carried out on peripheral whole blood without taking any steps which would concentrate the fetal DNA. As pointed out in section 5 above, by using serum or plasma in accordance with the present invention, instead of whole blood, fetal DNA concentration is increased by at least 20 times. (The actual figure is likely to be higher as one is comparing plasma of the first trimester with whole blood in the second trimester and it is known that fetal DNA concentration increases from the first to second trimester. "At least 20" therefore appears highly conservative.) The delta-globin gene experiment (b) relates to the third trimester of pregnancy, but the concentration of fetal DNA in the maternal whole blood at this time would still be less than the concentration of fetal DNA present in maternal serum or plasma during the

first trimester. I say this because the experiments relating to the present invention show that the concentration of fetal DNA in plasma or serum rises by a factor of about 12 times from the first to the third trimester of pregnancy (see Figures 4A and 4B) of the present patent application), whereas the concentration of fetal DNA in the maternal whole blood (from which the DNA was isolated) in the first trimester is much higher than 12 times that of plasma, at the lowest estimate at least 20 times higher.Therefore, the experimental showing of the delta-globin gene experiment (b) that one can detect DNA in maternal whole blood by a PCR technique in the third trimester of pregnancy at a concentration "c", suggests that the same PCR technique would be able to detect it in maternal plasma in the third trimester, where I believe it would be present at a concentration of at least "20c" and in maternal plasma in the first trimester, where I believe it would be at a concentration of at least "20c/12" = at least 1.6 times "c". In other words, if the PCR technique used in the literature can detect the DNA at concentration "c", it must be able to detect it at 1.6 times that concentration. This comparison assumes that the ratio of fetal DNA in maternal plasma or serum to fetal DNA in whole maternal blood does not differ greatly according to the stage of pregnancy, but it also incorporates the highly conservative assumption that the ratio might be as low as 20:1. I believe therefore that the comparison is reasonable. Thus, it is credible to me that the method of the present invention, using the known PCR techniques of restriction enzyme digest and "ARMS", on maternal plasma or serum, would enable small genetic changes to be detected. The present specification does not mention these techniques specifically, but I believe that any competent colleague in this field would appreciate that if simpler PCR techniques do not work well in relation to detecting any particular type of genetic deviation in fetal DNA in maternal serum or plasma, one would resort to the use of published more sophisticated techniques such as are described in this paper.

(c) Saito *et al.*, The Lancet **356**, 1170 (2000) describe the diagnosis of fetal achondroplasia, a genetic defect which gives rise to short limbs. Although it can be inherited as an autosomal disorder, most of the cases are sporadic (l.h. column, lines 11-13 from bottom). In nearly all cases, genetic defect is a single nucleotide change in the gene encoding human fibroblast growth factor receptor 3. This gene has been mapped to chromosome 5. The paper describes detecting this defect in a fetus at 30 weeks of pregnancy, by PCR amplification of DNA in maternal plasma. The mother was not suffering from this disease. Ultrasonography suggested that her

fetus might be. The PCR amplification was carried out using primers, known in 1994 from quoted reference (4) of the paper. The defect creates a restriction enzyme site not present in the normal allele. Restriction fragment analysis of the PCR product was therefore carried out. The mutant allele was detected in the maternal plasma and confirmed from a fetal amniotic sample.

(d) Amicucci *et al.* referred to above in section 3 of this Declaration.

(e) William Reed *et al.* have a poster displayed on the worldwide web, the title of which is "Non-invasive Prenatal HLA Typing of a Sibling Cord Blood Donor through Kinetic PCR Analysis of Maternal Plasma." (see http://www.abstracts-on-line.com). HLA denotes a human leukocyte antigen, which is a glycoprotein encoded on chromosome 6. The paper points out the importance of knowing whether the fetus is of the correct HLA type if its cord blood is to be used as a source of stem cells for transplantation. These authors carried out kinetic PCR on the maternal blood successfully to detect paternally inherited HLA alleles, the mother having been found to be free of such alleles. Kinetic PCR involves the coupling of a PCR reaction to the generation of a fluorescent signal and the monitoring of the resultant change in emitted fluorescence. It has been described before the priority date of the present application in European Patent Application 512,334 and also in Higuchi *et al.*, Biotechnology (NY) **11**, 1026-1030 (1993).

Conclusory Statements

7. I believe that the above examples show that when the competent person in the field has been taught, by the present invention, that the maternal plasma or serum contains sufficient fetal DNA in the high concentrations found, he would have no difficulty in carrying out fetal diagnosis to detect even small genetic defects, in any chromosomal locus, and even in the first trimester of pregnancy. Suitable amplification techniques can be ordinary PCR or more sophisticated developments thereof, but these techniques were all known in the literature before the date of my invention. As shown particularly by the Saito *et al.* reference in section 5 above, even a point mutation is detectable.

8. It is not necessary for the success of the method of the present invention that the gene to be detected be paternally inherited. If the mother does not show the disease phenotype, that is sufficient. If the mother can be demonstrated not to have the gene defect for which the fetus is being tested, either through not having the disease phenotype or by prior genetic testing, it matters not whether the gene to be tested is paternally inherited: appearance of the fetal gene in the maternal plasma or serum will indicate its fetal origin.

8. Indeed, the present invention can be used to diagnose Down's syndrome in a fetus, by carrying out the invention on maternal plasma. The diagnosis depends on detecting an abnormally strong signal from DNA sequences present on chromosome 21 (three copies of which are carried by the fetus). See Poon *et al.*, The Lancet **356**, 1819-1820 (November 25, 2000). The defect giving rise to an additional copy of chromosome 21 is believed to be maternal in origin. At all events, this a case in which it is irrelevant how it is inherited, as the additional copy in fetal DNA can be distinguished, if necessary using as a marker a probe for another chromosome.

Veracity Declaration

9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon.

Y-M DENNIS LO

22 APRIL 2002

Date